Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5088–5092, June 1992
Biochemistry

Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription

(two-component system/nitrogen regulation/phosphorylation/cooperative interaction)

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Contributed by Boris Magasanik, March 9, 1992

ABSTRACT **We studied the effect of phosphorylation of nitrogen regulator I (NR_I) on its binding properties. Both phosphorylated and unphosphorylated NR_I bind linearly to a single binding site but cooperatively to two adjacent binding sites. Cooperative binding of NR_I is severely affected by phosphorylation: half-maximal binding of NR_I-phosphate is at 20-fold lower concentrations than that of unphosphorylated NR_I. This is more due to a huge increase in the cooperativity constant—which is the strength of interaction between two NR_I dimers—than to an increase in the microscopic binding constant which is the binding affinity to a single binding site. *In vitro* transcription and DNA footprinting experiments showed that occupation of a single binding site by NR_I is not enough for efficient activation and that activation only occurs at a higher NR_I concentration. We propose an activation mechanism for NR_I in which the phosphorylation of NR_I induces a conformational change in the N-terminal domains of the NR_I-phosphate dimers, which now interact strongly with each other, leading to a tetramerization of NR_I upon binding to two adjacent binding sites. We propose that not the phosphorylation of NR_I itself but rather the tetramerization of NR_I-phosphate on DNA binding induces the conformational change of the central domain to the active conformation.**

Two-component systems that enable bacteria to adapt efficiently to changes in the environment consist of two proteins—the transmitter, a protein kinase, and the receiver, the response regulator, which in most cases is a transcriptional activator (reviewed in refs. 1 and 2).

One of the best-studied systems is the one responsible for control of the expression of genes in response to the availability of a nitrogen source (reviewed in ref. 3). In this case the kinase, nitrogen regulator II (NR_{II}), responds to nitrogen deprivation by phosphorylating nitrogen regulator I (NR_I); NR_I-phosphate binds to sites usually situated ≈100 base pairs (bp) upstream of the nitrogen-regulated promoters and catalyzes the conversion of a σ^{54}–RNA polymerase promoter closed complex to the open complex. The core of this system is the *glnALG* (*glnA ntrBC*) operon with genes coding, respectively, for glutamine synthetase, NR_{II}(NtrB) and NR_I(NtrC).

Three strong NR_I binding sites are associated with this operon (4, 5). Two of these sites (sites 1 and 2), situated on the same face of the DNA helix with a center-to-center distance of 31 bp, overlap the σ^{70}-dependent promoter *glnAp1* and are 100 bp distant from the σ^{54}-dependent promoter *glnAp2*. A third NR_I binding site (Lp) overlaps the σ^{70}-dependent promoter *glnLp*. It has been shown that in cells growing with excess nitrogen, the transcription of *glnA* (the structural gene for glutamine synthetase) initiates at *glnAp1*, the transcription of *glnG* (the structural gene for NR_I) initiates at *glnLp*, and both promoters are subject to negative control by NR_I. A shift of the cells to a nitrogen-deficient medium results in phosphorylation of NR_I and activation of the initiation of transcription at the strong *glnAp2* promoter. The resulting increase in the intracellular concentration of NR_I causes a complete block of transcription initiation at *glnAp1* and *glnLp* so that, in nitrogen-deficient cells, transcription initiation at *glnAp2* is solely responsible for synthesis of glutamine synthetase and of NR_I. The initiation of transcription at *glnAp2* is activated by NR_I-phosphate bound to sites 1 and 2 (3).

Previous studies indicated cooperative interaction between NR_I or NR_I-phosphate bound to the two upstream binding sites of the nifLA promoter (6). It has also been reported that NR_I-phosphate binds more strongly than NR_I to DNA (6, 7). In the current study, we compare the affinities of NR_I and of NR_I-phosphate for single binding sites with their affinities for sites 1 and 2 located upstream from *glnAp2* and correlate these measurements with the ability of NR_I-phosphate to activate transcription at *glnAp2*. Our results indicate that this ability depends on the cooperative interaction of NR_I-phosphate dimers.

MATERIALS AND METHODS

Proteins. NR_I and NR_{II}2302 were prepared as described (8, 9). The concentrations of NR_I and NR_{II}2302 were estimated from their absorbances at 280 nm, using $A^{1\%} = 9.1$ for NR_I and $A^{1\%} = 4.15$ for NR_{II} (10). In contrast to previous studies (11, 12), the concentrations of NR_I are expressed in terms of the dimer. Core RNA polymerase and σ^{54} were purified as described (9).

Oligonucleotides. All binding sites were synthesized as 26-bp oligonucleotides and were radioactively end-labeled with the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs). End-labeled DNA was separated from unincorporated nucleotides by polyacrylamide gel electrophoresis. For the experiments with the adjacent binding sites 1 and 2 of *glnAp2*, the 173-bp *Eco*RI/*Sph* I fragment from pVW4 carrying these sites was purified by polyacrylamide gel electrophoresis.

Filter Binding Assay. [^{32}P]DNA was incubated with various amounts of NR_I in the presence or absence of 30 nM NR_{II}2302 at 20°C in standard buffer (50 mM Tris·HCl/50 mM KCl/5 mM $MgCl_2$/0.1 mM EDTA/2 mM ATP/100 μg of bovine serum albumin per ml) for 10–15 min in a total vol of 60 μl to 1 ml. DNA concentration was ≈0.5 nM for binding experiments with a single binding site, site 1 or 2, and ≈0.5 pM in

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Abbreviations: NR_I, nitrogen regulator I; NR_{II}, nitrogen regulator II.

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all other binding experiments. Then, 50- to 800-μl aliquots were filtered through nitrocellulose filters (Schleicher & Schuell; 2.5 cm) and immediately washed with 400 μl of standard buffer without ATP and bovine serum albumin. Samples were filtered and washed in <1 s under suction. Filters were dried and assayed by liquid scintillation counting.

Calculation of Kinetic Parameters. Calculations were done according to Ackers and coworkers (13, 14), using $Y = K[L]/(1 + K_1[L])$ for the binding of ligand to a single binding site, where Y is the fraction of DNA to which ligand has bound and [L] is the free ligand concentration, which equals the total ligand concentration at [L] >> [DNA]. K is the association constant of ligand to the DNA.

The binding isotherm for binding of ligand to DNA with two binding sites for the ligand with the microscopic binding constants K_1 and K_2 and a cooperative interaction constant of $K_{1,2}$ is given by $Y = x/(1 + x)$ where $x = (K_1[L] + K_2[L] + K_1K_2K_{1,2}[L]^2)$.

Construction of Transcription Templates. All plasmids were derived from vector pTE103, which contains a multicloning site placed upstream from a strong T7 transcription terminator (15). Plasmid pAN6 contains the *glnALG* regulatory region without the NR_I binding sites (7). Construction of pVW7 was as follows: the 124-bp *Sal* I fragment carrying sites 1 and 2 of *glnAp2* from pAN7 (7) was subcloned into the *Sal* I site of pUC19, generating pVW4. Ligation of the small *Pst* I/*Eco*RI fragment from pVW4 to the large *Pst* I/*Eco*RI of pAN6 (7) created pVW7, in which sites 1 and 2 of *glnAp2* are located 1 bp further upstream from the promoter than in the wild type. Plasmid pVW9 was constructed as follows: the complementary synthetic oligonucleotides 5′-TACAGGTTGCACCATTTTAGTGCATT-3′ and 5′-TAAATGCACTAAAATGGTGCAACCTG-3′ were heated at 95°C and annealed by cooling at room temperature. The annealed oligonucleotides were ligated to the *Ase* I ends of pBR322 generating pVW6. Ligation of the 384-bp *Dra* I/*Pst* I fragment to the *Pst* I/*Eco*RI (filled in) ends of pAN6 created plasmid pVW8. pVW8 was cut with *Hin*dIII, filled in, and religated to generate pVW9, in which the center of the *glnLp* NR_I binding site is located 130 bp upstream from the *glnAp2* promoter. Transcription of *glnAp2* on plasmids pAN6, pVW7, and pVW9 generates a transcript of 484 nucleotides.

Transcription Assay. Transcription experiments were performed as described (11, 12, 16). Supercoiled DNA templates were purified by centrifugation in CsCl/EtdBr gradients. The concentration of plasmid DNA in the reaction mixture was 5 nM. Proteins were present at the following concentrations: core RNA polymerase, 25 nM; σ^{54}, 100 nM; NR_{II}, 15 nM; NR_I, as indicated. Transcripts were run in urea/acrylamide gels, visualized by autoradiography, and quantified as described (9, 12).

DNase I Footprinting. DNase I protection experiments of supercoiled DNA were carried as described (12, 17). Plasmid

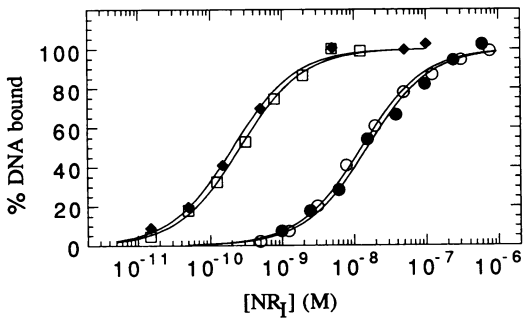

FIG. 1. Binding of NR_I to a single binding site. ●, Site 1; ○, site 2; ◆, site Lp; □, site 1′.

Table 1. Oligonucleotides containing NRI binding sites

Site 1	cgatTGCACCAacaTGGTGCttat
Site 2	cggaaGCACtAtatTGGTGCAaca
Lp	cgaaTGCACtAaaaTGGTGCAacc
Site 1′	ccatTGCACCAacaTGGTGCAtat
Consensus	TGCACCA——TGGTGCA

Nucleotides responsible for dyad symmetry are shown in capital letters. We number the consensus sequence from 1 to 17.

DNA was incubated with the proteins under the same conditions and with the same buffer used for the transcription assays. NR_I was added as indicated, and the mixtures were incubated for 20 min at 37°C. Annealing of the FC3 primer (5′-GGTCATGGTCGTCGTGG-3′), which hybridizes to *glnA* sequences at +54 (5′ position) (ref. 18; Fig. 1), was carried out at 50°C.

RESULTS

Binding of NR_I and NR_I-Phosphate to a Single Site. We measured the binding of NR_I to its binding sites by the nitrocellulose filter binding assay (19). We used DNA containing site 1 or 2, both of which are located upstream from the wild-type *glnAp2* promoter (4), and DNA containing the NR_I-binding site overlapping the *glnLp* promoter (5). We also used DNA containing site 1′, which differs from wild-type site 1 by the substitution of A for T in position 17 of the consensus NR_I binding sequence, resulting in a sequence with perfect dyad symmetry (Table 1).

Fig. 1 shows the binding of unphosphorylated NR_I to these sites as a function of the NR_I concentration. The data can be fit by simple hyperbolic isotherms (solid lines), which is expected, since the binding sites exhibit dyad symmetry and NR_I is a stable dimer in solution at concentrations as low as 5 nM (data not shown). Sites 1 and 2 are rather weak (half-maximal binding is at ≈14 nM); the Lp and 1′ binding sites are much stronger (half-maximal binding at ≈0.2 nM) (Table 2). Phosphorylation of NR_I, accomplished by adding NR_{II} to the mixture containing ATP and Mg^{2+}, did not alter its affinity for site 1 (Fig. 2). Due to the autophosphatase activity of NR_I-phosphate (10, 20), we found that only 10% of the NR_I is present as the phosphate in the reaction conditions used. We would therefore not have detected a 2-fold enhancement of binding ability resulting from the phosphorylation, but we would have been able to detect a 4-fold enhancement. Apparently, NR_I and NR_I-phosphate compete approximately equally well for binding to a single site.

Binding of NR_I and NR_I-Phosphate to Two Adjacent Sites. In these experiments, a 173-bp restriction fragment from the upstream region of *glnAp2* was used that contains NR_I binding sites 1 and 2. Based on the assumption that there is

Table 2. Affinity of NR_I-phosphate for different binding sites

	Half-maximal binding, nM	
Binding sites	NR_I	NR_I-phosphate
1	12.7	12.7
2	15.2	15.2
Lp	0.21	ND
1′	0.27	ND
1 + 2	2	0.1

In the case of single sites, half-maximal binding defines the dissociation constant K_d. It is equivalent to ΔG of approximately −10 kcal (1 cal = 4.184 J) at 20°C for sites 1 and 2, and of −12 kcal for sites Lp and 1′. Description of NR_I and NR_I-phosphate binding to sites 1 and 2 located on the same DNA requires, in addition to the dissociation constants for the single sites, the cooperativity constant $K_{1,2}$, which is 27 mM (ΔG = −2.1 kcal) for NR_I and 0.55 μM (ΔG = −8.4 kcal) for NR_I-phosphate.

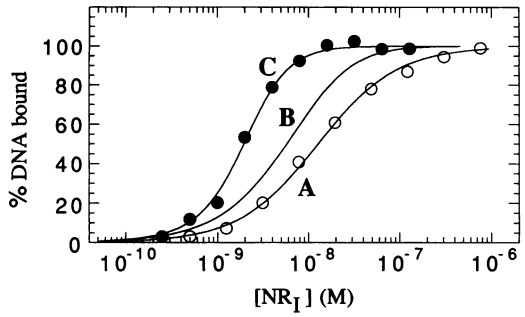

FIG. 2. Binding of phosphorylated NR_I to site 1. ●, Nonphosphorylated NR_I; ○, phosphorylated NR_I.

no interaction between the NR_I dimers bound to the two sites, we can calculate the expected binding curve from the microscopic binding constants for each of the two sites. The simulated binding isotherm (Fig. 3, curve B) shows a 2-fold increase in affinity (compare curve B with curve A). However, the actual results of the binding experiments, shown in curve C, reveal stronger binding than in curve B and a steeper binding isotherm, indicating cooperative binding of NR_I to the two sites. To fit the data points (solid line in curve C), we had to introduce a cooperativity constant of 27 mM (Table 2).

We next determined the effect of phosphorylation of NR_I on cooperative binding to two sites. The experimental results show that phosphorylation greatly increases the cooperative interaction (Fig. 4, compare curves C and B). It can be seen that phosphorylation has decreased the half-maximal binding from 2 to 0.1 nM. Phosphorylated NR_I has achieved maximal binding (curve C) at a concentration when NR_I (curve B) just begins to bind (Table 2). Assuming that the cooperative interaction involves two molecules of NR_I-phosphate and that NR_I and NR_I-phosphate bind equally well to a single binding site and considering that NR_I-phosphate constitutes only 10% of the total NR_I, we had to use a much higher cooperativity constant (0.55 μM) to fit the data points.

A mutation in *glnG* has resulted in NR_I316 with an alteration in the central domain. This altered protein has some ability to activate transcription in its unphosphorylated form and has greatly increased activating ability when phosphorylated (21). Apparently, the central domain in NR_I316 already exists partially in the active conformation. If the central domain were responsible for the strong cooperative interaction in NR_I-phosphate, we would expect a difference in the binding of unphosphorylated NR_I316 compared to wild-type NR_I. We found that the ability of NR_I316 and NR_I316-phosphate to bind to DNA with one or two sites does not differ from that of NR_I and NR_I-phosphate, respectively (data not shown). Apparently, the central domain is not the site responsible for the cooperative interaction.

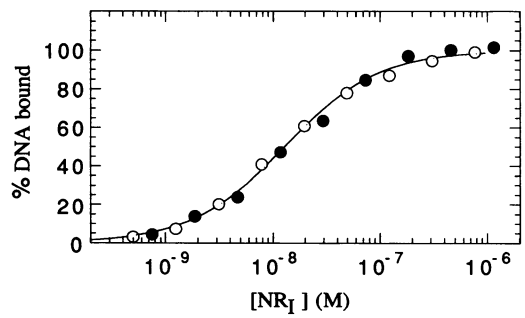

FIG. 3. Binding of NR_I to adjacent binding sites 1 and 2. Curves: A, single site 1; B, calculated independent binding to sites 1 and 2; C, actual binding to sites 1 and 2.

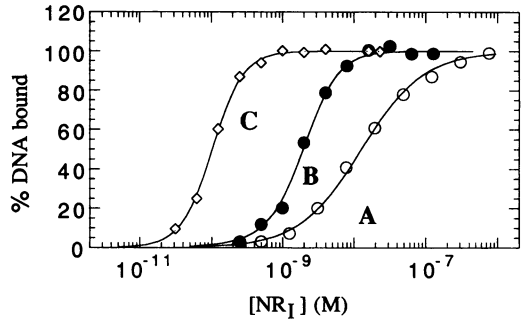

FIG. 4. Binding of phosphorylated NR_I to adjacent binding sites 1 and 2. Curves: A, nonphosphorylated NR_I to single site 1; B, nonphosphorylated NR_I to sites 1 and 2; C, phosphorylated NR_I to sites 1 and 2.

Activation of Open Complex Formation on Plasmid Templates Containing Two Adjacent Binding Sites, a Single Binding Site, or No Binding Site for NR_I. In these experiments, 5 nM plasmid DNA, a much higher concentration than that required for the nitrocellulose filter binding, had to be used. In this case, the binding to strong sites, such as site Lp or adjacent sites 1 and 2, is limited not by the strength of the site but by the amount of NR_I-phosphate; the DNA is titrated and, consequently, no direct comparison of the NR_I concentration required for activation of open complex formation and for half-maximal binding of DNA (presented in Table 2) can be made. We first compared the ability of phosphorylated NR_I to protect DNA from DNase I digestion in plasmids containing adjacent sites 1 and 2 (pVW7) or the single binding site Lp (pVW9), under the conditions used for the transcription assays. These footprinting experiments (Fig. 5) show that in either case, 5 nM NR_I provided partial protection, and

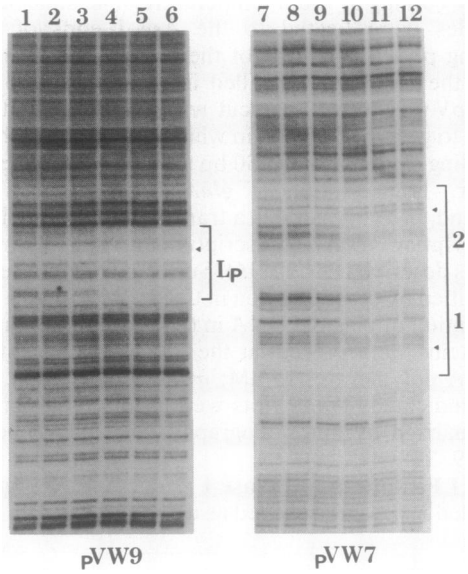

FIG. 5. Analysis of NR_I binding to two adjacent binding sites (pVW7) or to a single binding site (pVW9) by DNase I protection. Plasmid DNA was incubated with NR_I, NR_{II}, σ^{54}, and core RNA polymerase under the conditions used for transcription assays. Lanes: 1 and 7, no NR_I; 2 and 8, 2.5 nM; 3 and 9, 5 nM; 4 and 10, 12.5 nM; 5 and 11, 25 nM; 6 and 12, 50 nM. After brief digestion with DNase I, the DNA fragments were denatured and the ^{32}P-end-labeled FC3 primer was annealed and extended with Klenow. Reaction products were subjected to electrophoresis on a sequencing gel followed by autoradiography. Regions protected by NR_I are indicated by brackets. Bands hypersensitive to DNase I digestion are indicated with arrowheads. Products of the dideoxynucleotide sequencing reactions were also electrophoresed adjacent to lanes 1 and 12 (data not shown).

12.5 nM NR_I provided full protection. We noted, concomitant with the binding of NR_I to the two adjacent binding sites, an increased hypersensitivity to DNase I digestion at two positions in this region; in the case of the single binding site, DNase I sensitivity at a corresponding site only appeared at an NR_I concentration greatly in excess of that required for protection (Fig. 5).

We found that the ability of NR_I-phosphate to activate open complex formation bound to sites 1 and 2 or to the single Lp site was totally different. As shown in Fig. 6, with the template carrying sites 1 and 2, open complex formation was half-maximal at an NR_I concentration of ≈2.5 nM, and almost maximal at a concentration of 5 nM; but in the template containing the single Lp site, there was no open complex formation at an NR_I concentration of 5 nM, and maximal open complex formation required NR_I at 25 nM concentration. Apparently the full occupation of two adjacent sites but not of a single site results in maximal open complex formation. It is of interest that in both instances open complex formation is coordinated with the appearance of hypersensitivity to DNase I digestion at corresponding positions of the double and the single sites (Fig. 5).

We considered the possibility that the almost complete inability of NR_I to activate transcription at a concentration when it fully occupies the single site may result from the equal affinity of NR_I and NR_I-phosphate for a single site (see Fig. 2). Since the majority of the NR_I is not phosphorylated, only a small fraction of the DNA templates will be occupied by NR_I-phosphate, which may be sequestered in the open RNA polymerase promoter complex. We therefore altered our experimental procedure to produce, instead of the open complex, an elongation complex consisting of DNA, σ^{54}–RNA polymerase, and an RNA oligomer of 18 nucleotides (16). We had shown previously that in this case NR_I can move readily from its binding site to that of other DNA templates to activate transcription (16). We find now that the response to NR_I concentration in the case of the template with the single binding site is exactly the same whether open complex formation (as in the experiment shown in Fig. 6) or elongation complex formation (data not shown) is measured. This observation militates against the view that sequestration of NR_I accounts for the failure of NR_I in low concentration to activate transcription from the template with the single binding site. NR_I-phosphate can repeatedly dissociate from one template and associate with another to activate transcription. Furthermore, competition of NR_I and NR_I-phosphate for binding to the single site would not explain the sharp increase in open complex formation resulting from the increase in the NR_I concentration from 10 to 20 nM (Fig. 6*B*).

It is of interest that activation of transcription is also seen when a template devoid of NR_I binding sites is used; in this case, open complex formation is first noticeable at a NR_I concentration of 25 nM and maximal open complex formation requires a 100 nM concentration (Fig. 6*A*).

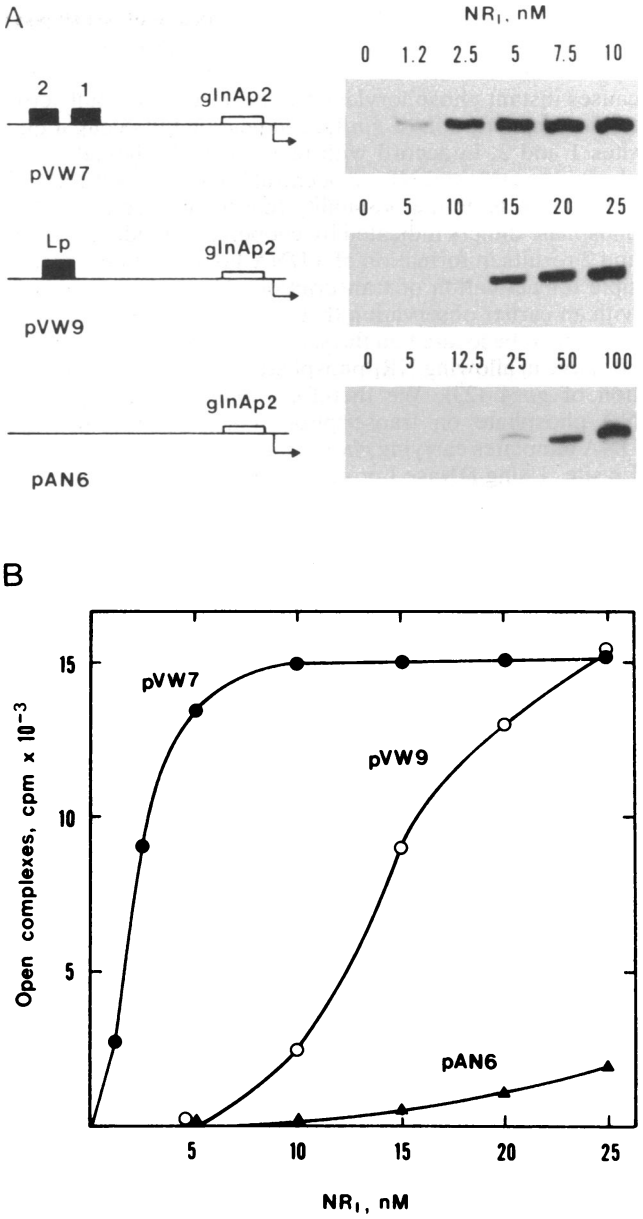

FIG. 6. Transcriptional activation of *glnAp2* by NR_I-phosphate on plasmid templates carrying two adjacent NR_I binding sites (pVW7), a single site (pVW9), or no sites (pAN6). (*A*) Concentrations (nM) of NR_I (shown above each lane) required to activate open complex formation. (*B*) Quantitation of transcriptional activation by NR_I. Radioactive transcripts were cut out of gels and radioactivity was measured. Average of three experiments is shown. A relative activity of 100% corresponds to 15,000 cpm.

DISCUSSION

We have shown that NR_I and NR_I-phosphate bind approximately equally well to single sites and that the site overlapping *glnLp* has much greater affinity for NR_I than either site 1 or site 2. As shown in Table 1, the binding sites for NR_I, a dimer in solution, consist of two 7-bp-long inverted repeats with dyad symmetry. A mutation in position 17 of site 1 results in site 1′, with perfect dyad symmetry and greatly increased affinity for NR_I. The Lp site has an affinity for NR_I equal to that of site 1′ and differs from this site by having a T rather than a C in position 6. Apparently, this deviation from perfect symmetry does not affect the binding of NR_I, in contrast to the deviation resulting from an A in position 1 of site 2 or a T in position 17 of site 1.

It can be seen that there is cooperative binding of NR_I to sites 1 and 2. Nevertheless, the affinity of the Lp site for NR_I still is 10-fold greater than that of sites 1 and 2 together. However, phosphorylation of NR_I causes a huge increase (≈50,000-fold) in the cooperativity constant and, therefore, the affinity of NR_I-phosphate for sites 1 and 2 that is similar to the affinity for the Lp site (Table 2).

These observations are in excellent accord with earlier determinations of the intracellular concentration of NR_I in cells grown with excess nitrogen, of the inhibition of expression initiated at the Lp promoter in these cells, and of the effect of NR_I phosphorylation on activation of transcription at *glnAp2*. NR_I is present in these cells at 1–2 nM (8), which according to our present demonstration of half-maximal binding of NR_I to the Lp site at 0.2 nM should lead to considerable, but not total, blockage of transcription initia-

tion at this promoter; by actual measurement, expression from this promoter is 5-fold lower than in cells lacking NR_I (22). A shift of such cells to a nitrogen-limited medium, which causes instant phosphorylation of NR_I, results in full activation of transcription of *glnAp2*, indicating full occupation of sites 1 and 2, in accord with half-maximal binding of NR_I-phosphate at a total NR_I concentration as low as 0.1 nM (4).

We considered the possibility that the interaction of NR_I-phosphate dimers indicated by cooperative binding to sites 1 and 2 results in formation of a DNA-bound tetramer responsible for activation of transcription. This view is in accord with an earlier observation that in intact cells binding sites 1 and 2 must be located on the same face of the helix to be fully effective in allowing NR_I-phosphate to activate the transcription of *glnA* (23). We therefore compared the effects of NR_I-phosphate on transcription initiation on supercoiled DNA templates carrying *glnAp2* and either sites 1 and 2 or the Lp site. Using DNase I protection experiments to determine occupancy of these sites by NR_I under the conditions of the transcription assays, we found that sites 1 and 2 and the Lp site become fully occupied at approximately the same concentration of NR_I (Fig. 5). This concentration of NR_I, $\approx$10 nM, was adequate for maximal activation of transcription in the case of the template carrying two NR_I-binding sites but was almost completely ineffective in the case of the template carrying a single binding site. Increasing the NR_I concentration to $\approx$20 nM did, however, bring about maximal activation of open complex formation on the template carrying a single binding site. Furthermore, when a template devoid of NR_I-binding sites was used, full activation could be achieved only at an NR_I-binding sites concentration of $\approx$100 nM (Fig. 6).

In all three cases, the curves describing the response to an increase in NR_I concentration are sigmoidal. This suggests that in all cases there is a cooperative interaction of two NR_I dimers and that only the tetramer is capable of activating open complex formation at *glnAp2*. We assume that, in the case of a single binding site, an increase in the concentration of NR_I facilitates interaction of the NR_I-phosphate dimer bound to DNA with another dimer, resulting in formation of a tetramer, with only one of the dimers firmly anchored to DNA. The increased hypersensitivity to DNase I digestion at a position within the binding site for NR_I, which becomes apparent at the NR_I concentration adequate for activation of transcription with two or a single binding site, may be the signature of the cooperative interaction (Fig. 5). At a very high concentration of NR_I, tetramers might form in solution and, without binding to DNA or by nonspecific binding to DNA, bring about conversion of the closed to the open σ^{54}–RNA polymerase *glnAp2* complex. This view is in accord with the results reported by Weiss *et al.* (24). These authors have shown that phosphorylation endows NR_I with an ATPase activity that is essential for its ability to activate transcription. Their experiments demonstrate cooperativity in the response of ATPase activity to an increase in the concentration of NR_I-phosphate, with maximal activity at a NR_I-phosphate concentration corresponding approximately to that required in our experiments to fully activate transcription on a template without binding sites.

In a general discussion of "two-component regulatory systems," Kofoid and Parkinson (2) have suggested that phosphorylation of a receiver results in its dimerization to produce the active conformation. In keeping with this idea is the observation that all known NR_I-activatable promoters are endowed with two NR_I binding sites located on the same face of the helix. This has been shown by binding experiments in the case of *glnAp2* (4), *glnHp2* (12), and *nifLA* (6). In the case of *hisJp* (also called *dhuA*), binding of NR_I to a single site located 165–182 bp distant from the translational start site has been demonstrated (25), but inspection of the nucleotide sequence reveals the presence of a second site at 195–211 bp (26). Similarly, sequence analysis has revealed two NR_I-binding sites located upstream of the promoter for the *nac* gene (ref. 27; R. A. Bender, personal communication).

We propose that interaction of the phosphorylated N-terminal domains of NR_I results in a conformational change in the central domains of the dimers bound to DNA and that the actual activator of transcription is the NR_I-phosphate tetramer.

We thank Alexander Ninfa for purified NR_{II}2302 and Hilda Harris-Ransom for typing the manuscript. Oligonucleotide synthesis was performed at the Biopolymers Laboratory, Howard Hughes Medical Institute, Massachusetts Institute of Technology. This work was supported by Grant DMB-8817091 from the National Science Foundation.

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